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# Glutathione Transferases in Herbicide-Resistant and Herbicide-Susceptible Black-grass (*Alopecurus myosuroides*)*

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**Abstract:** Glutathione transferase (GST) activities toward the selective herbicide fenoxaprop-ethyl, together with thiol contents, have been compared in seedlings of wheat (*Triticum aestivum*) and two populations of black-grass (*Alopecurus myosuroides*) which are resistant to a range of herbicides (Peldon and Lincs E1), and a black-grass population which is susceptible to herbicides (Rothamsted). GST activities toward the non-cereal herbicides metolachlor and fluorodifen were also determined. On the basis of enzyme specific activity, GST activities toward fenoxaprop-ethyl in the leaves were in the order wheat > Peldon = Lincs E1 > Rothamsted, while with fluorodifen and metolachlor the order was Peldon = Lincs E1 > Rothamsted > wheat. Using an antibody raised to the major GST from wheat, which is composed of 25-kDa subunits, it was shown that the enhanced GST activities in both Peldon and Lincs E1 correlated with an increased expression of a 25-kDa polypeptide and the appearance of novel 27-kDa and 28-kDa polypeptides. Leaves of both wheat and black-grass contained glutathione and hydroxymethylglutathione, with the concentrations of glutathione being in the order Peldon > Lincs E1 = Rothamsted = wheat. However, in glasshouse dose-response assays, the Lincs E1 population showed much greater resistance to fenoxaprop-ethyl than Peldon. We conclude that high GST activities and the availability of glutathione may contribute partially to the relative tolerance of black-grass to herbicides detoxified by glutathione conjugation. Although herbicide-resistant populations show enhanced GST expression, in the case of fenoxaprop-ethyl the associated increased detoxifying activities alone cannot explain the differences between populations in the degree of resistance seen at the whole plant level.

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## 1 INTRODUCTION

Glutathione transferases, also referred to as glutathione *S*-transferases (GSTs, EC 2.5.1.18), catalyse nucleophilic substitution, or, more rarely, addition, reactions between the tripeptide glutathione (gamma-glutamyl-cysteinyl-glycine) and electrophilic compounds of man-made or natural origin.[1] GSTs in crops are important in detoxifying major classes of herbicides, including chloro-*s*-triazines, chloroacetanilides, sulfoxide derivatives of thiocarbamates, diphenyl ethers and several aryloxyphenoxypropionate and sulfonylurea herbicides.[2] The diverse and abundant GSTs in maize (*Zea mays* L.) have been relatively well characterised at the biochemical and molecular level.[1] GSTs with activity toward herbicides are also present in other cereals such as sorghum (*Sorghum bicolor* L.) and wheat (*Triticum aestivum* L.), but far less is known regarding the importance of these enzymes in conferring herbicide tolerance in these species.[4] The observation that the selectivity of the aryloxyphenoxypropionate herbicide fenoxaprop-ethyl is determined by rapid detoxification by glutathione conjugation in wheat and slower rates of conjugation in susceptible grass weeds has been of particular interest.[5] Glutathione conjugation mediated by GSTs has a well-defined role in the selectivity of chloroacetanilide and chloro-*s*-triazine herbicides in maize and associated weeds,[4] but the importance of these enzymes in fenoxaprop-ethyl detoxification is not clearly understood.[6] In recent studies we have identified GST activities toward fenoxaprop in species of *Triticum*, including *T. aestivum*, which can be induced by herbicide safeners[7] and have purified several isoenzymes from the shoots of safener-treated wheat seedlings.[8] However, to define a role for GSTs in the selectivity of herbicides which owe their selectivity in wheat to glutathione conjugation,[5,9,10] it is necessary to examine the spectrum of GSTs present in competing weeds. As part of this programme we became interested in the GST activities of the problem grass weed black-grass (*Alopecurus myosuroides* Huds.), particularly as many populations of this weed in the UK have been found to be resistant to a range of herbicides, including fenoxaprop-ethyl.[11] Since GSTs have been implicated in the development of resistance to chloro-*s*-triazine herbicides in populations of the maize weed *Abutilon theophrasti* (L.) Medic.,[12] it also seemed possible that GSTs were involved in resistance to fenoxaprop-ethyl in black-grass.

To test this hypothesis, we have determined GST activities toward fenoxaprop-ethyl, the diphenyl-ether fluorodifen and the chloroacetanilide metolachlor, together with thiol contents in wheat, and compared them with those determined in populations of black-grass which differ in their sensitivity to several herbicides.[11,13,14] Metolachlor and fluorodifen were included, as examples of chloroacetanilides, are known to be detoxified by glutathione conjugation in wheat,[10] while fluorodifen is an excellent herbicide substrate of wheat GSTs.[7] The Rothamsted population of black-grass was used as an example of the herbicide-susceptible wild-type weed. The herbicide-resistant populations, Peldon and Lincs E1, showed differential sensitivities to chlorotoluron and fenoxaprop-ethyl in glass-house trials.[11,13,14] The resistance indices (the ratios of $ED_{50}$ values relative to a susceptible standard) demonstrated that, with chlorotoluron, Peldon (resistance index 27.6) was more resistant than Lincs E1 (2.6), while the values were almost exactly the reverse with fenoxaprop-ethyl, Lincs E1 (27.2) being much more resistant than Peldon (3.9).

## 2 MATERIALS AND METHODS

### 2.1 Plant material and growth conditions

Seeds of wheat (*T. aestivum* cv. Hunter) were obtained from Plant Breeding International, Cambridge, UK. Seeds were collected from the black-grass (*A. myosuroides*) populations Peldon, Lincs E1 and Rothamsted.[11,13,14] Prior to sowing, seeds were placed on wet filter paper in Petri dishes and incubated under ambient conditions on the bench. After three days the seeds were spread evenly over pre-wetted Levington compost in seed trays and then covered with compost (1 cm deep). The plants were maintained in an environmental growth chamber at 25°C under cool-white fluorescent lighting (510 $\mu$mol $m^{-2}$ $s^{-1}$) with a 16-h photoperiod. At 10, 20 and 30 days after first imbibing the seed, the foliage of the plants was collected by cutting the stems at ground level. Plants were sampled in duplicate, with each replicate consisting of a whole seed tray of approximately 200 plants. The foliage was weighed and then frozen in liquid nitrogen and stored at −80°C.

### 2.2 Assay for GSTs

Frozen foliage was ground to a powder in liquid nitrogen using a pestle and mortar and then extracted with Tris HCl (0·1 M; pH 7·5) containing EDTA, (2 mM), dithiothreitol (1 mM) and polyvinylpolypyrrolidone (50 g $kg^{-1}$). After straining through muslin the extract was centrifuged (10 000*g*, 10 min) and after decanting, ammonium sulfate was added to the supernatant to 80% saturation. The protein pellet was collected by centrifugation and then desalted in Tris HCl (20 mM; pH 7·5) using Sephadex G-25 PD-10 columns, as recommended by the manufacturer (Pharmacia). Protein content was determined using the Biorad dye-binding

assay kit with gamma-globulin for reference and the extracts from the various sources adjusted to identical protein concentrations. The extracts were then assayed for GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) using a spectrophotometric assay.[7] GST activity toward the herbicides was determined by incubating them with the crude plant extracts and glutathione and then quantifying the reaction products by HPLC as described previously,[7] with the exception that fenoxaprop-ethyl, rather than fenoxaprop, was used.

Extracts were also analysed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide gels.[15] The resolved polypeptides were electroblotted onto nitrocellulose membranes and then the blots washed using standard procedures.[16] The blots were then incubated with a 1000-fold diluted antiserum preparation from a rabbit which had been immunised with the major GST, composed of two 25-kDa subunits, purified from the shoots of wheat treated with the herbicide safener fenchlorazole-ethyl.[8] After rewashing the blot, the bound antibody was detected using a goat anti-rabbit IgG antibody coupled to alkaline phosphatase, after incubating with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.[16] For quantification of immunoreactive polypeptides, images of the blots were digitised with a Hewlett-Packard Scanjet II cx scanner and the integrated density of the stained bands determined using the UTHSCSA *Image Tool* programme.

### 2.3 Glutathione analysis

Plant tissue (1 g) was ground to a fine powder in liquid nitrogen and then allowed to thaw in hydrochloric acid (0·1 M; 3 ml). After 30 min on ice the slurry was centrifuged (10 000*g*, 5 min) and 100 $\mu$l transferred to each of two microcentrifuge tubes. Water (10 $\mu$l) was added to the first tube and glutathione (1 mM; 10 $\mu$l) added to the other. The two extracts were then processed in parallel through the remaining procedure. After the addition of NaOH (1 M; 10 $\mu$l), $NaBH_4$ (20 mg ml$^{-1}$, in 1 M NaOH; 10 $\mu$l) was added and each tube incubated for 10 min. The remaining $NaBH_4$ was then destroyed by adding hydrochloric acid (3·6 M; 10 $\mu$l). After centrifuging as above, 100 $\mu$l of the supernatant was transferred to a fresh tube and monobromobimane (10 mg ml$^{-1}$ in acetonitrile; 10 $\mu$l) was added, followed by *N*-ethyl-morpholine + water (35 + 65 by volume; 10 $\mu$l). The mixture was incubated in the dark for 20 min and the reaction was then stopped with acetic acid + water (5 + 95 by volume; 880 $\mu$l) and the sample re-centrifuged. A sample of the supernatant (50 $\mu$l) was injected onto a C-18 HPLC column (Phase Sep Cartridge S5ODS2, 250 × 4·6 mm) and eluted at 1 ml min$^{-1}$ with solvent A + solvent B (85 + 15 by volume), where solvent A was 50 mM $K_2HPO_4$ adjusted to pH 6 with acetic acid and solvent B was methanol. The eluent was monitored for fluorescence between 430 and 470 nm after excitation at 305–395 nm. The HPLC was calibrated by derivatising known amounts of glutathione in the range 0–20 nmol and samples corrected for losses during sample preparation using the 10 nmol spike of authentic thiol as an internal standard to monitor recovery.

## 3 RESULTS

### 3.1 GST activities in herbicide-resistant and herbicide-susceptible populations of black-grass

Wheat and black-grass were grown in an environmental chamber for 20 days and 30 days, respectively, until they were 8 cm high, the difference in sampling times reflecting the respective germination and growth rates of the two species. GST activities were then determined toward the model substrate CDNB and the herbicides fenoxaprop-ethyl, fluorodifen and metolachlor. Fenoxaprop-ethyl was used rather than fenoxaprop to increase the sensitivity of the assay method. The GST-mediated rate of glutathione conjugation of fenoxaprop-ethyl is 10 times less than with fenoxaprop.[7] However, when using fenoxaprop as substrate, a considerable proportion of the herbicide is consumed by rapid non-enzymic conjugation with glutathione and this substrate depletion reduces the accuracy of determination of the true enzymic rate. In contrast, the non-enzymic rate of conjugation of fenoxaprop-ethyl was considerably slower and this facilitated the accurate determination of the enzymic rate.

GST activities toward CDNB were slightly higher in the resistant black-grass populations than in wheat or the susceptible Rothamsted strain (Table 1). With fenoxaprop-ethyl as substrate, GST specific activities were higher in wheat than in the weeds, but, while no activity could be determined in the Rothamsted strain, both herbicide-resistant populations contained measurable activities toward the selective herbicide. Enzyme-specific activities toward fluorodifen and metolachlor were appreciably higher in black-grass than in wheat, with the extracts from Peldon and Lincs being more active than those from Rothamsted.

The enzyme activity data (Table 1) showed that the herbicide-resistant black-grass populations contained enhanced levels of GST activity relative to the susceptible black-grass, but did not indicate whether this was due to the increased expression of existing GSTs or the expression of novel isoenzymes. To examine these possibilities, polypeptides present in the three black-grass

**TABLE 1**
GST Activities in the Foliage of 20-Day-Old Wheat and Three Populations of 30-Day-Old Black-Grass

| *Species/Population* | *GST activity (nkat $g^{-1}$ protein) ($\pm SD$)*[a] | | | |
|---|---|---|---|---|
| | *CDNB*[b] | *Fenoxaprop-ethyl* | *Fluorodifen* | *Metolachlor* |
| Wheat | 5684 (±241) | 1·1 (±0·1) | 1·0 (±0·1) | 2·4 (±0·2) |
| Black-grass | | | | |
| Rothamsted | 4806 (±674) | 0 | 2·1 (±0·1) | 6·3 (±0·9) |
| Peldon | 8930 (±770) | 0·3 (±0·1) | 3·0 (±0·2) | 9·7 (±0·3) |
| Lincs E1 | 7618 (±634) | 0·4 (±0·1) | 2·8 (±0·3) | 8·4 (±0·1) |

[a] Enzyme activities refer to the mean of triplicate determinations.
[b] 1-chloro-2,4-dinitrobenzene.

populations were separated by SDS-PAGE and Western blots probed with an anti-wheat GST-serum. This antibody had been raised to the major GST present in untreated wheat shoots and wheat shoots from seedlings treated with the safener fenchlorazole-ethyl and is composed of two 25-kDa subunits.[8] Pre-immune serum failed to react with any of the black-grass polypeptides (data not shown). The anti-GST-serum strongly recognized a 25-kDa polypeptide in wheat corresponding to the respective GST subunit (Fig. 1). The antiserum also recognized a similar 25-kDa polypeptide in extracts from the herbicide-susceptible Rothamsted and showed a weak reaction toward a 27-kDa polypeptide, which, though not clearly visible in Fig. 1, could be determined on the original blots. In the extracts from Peldon and Lincs the antiserum reacted more strongly with the 25-kDa polypeptide and also clearly recognised additional polypeptides of 27 kDa and 28 kDa. Although the recognition by the anti-wheat GST-serum cannot be taken as absolute proof of the identity of these polypeptides, their relative molecular masses and the specificity of this antiserum for GSTs in other weed species (Hatton, P. J. pers. comm.), supports the assertion that the 25-kDa, 27-kDa and 28-kDa immunoreactive polypeptides in black-grass are all GST subunits.

To determine whether or not the relative expression of black-grass GSTs was affected by plant age, GST activities toward CDNB and immunoreactive polypeptides were assayed as detailed above in wheat and black-grass at 10, 20 and 30 days after seed imbibition (Table 2). Between day 10 and day 20, the specific GST activities in wheat increased nearly two-fold and this was associated with an approximate doubling in the immunoreactive 25-kDa subunit. In contrast, in all three populations of black-grass, the GST activities and expression of immunoreactive polypeptides was not significantly affected by age and identical to those given in Fig. 1 and Table 1.

## 3.2 Determination of glutathione content in wheat and black-grass populations

The availability of glutathione has been shown to be positively correlated with the selectivity of fenoxaprop-ethyl in wheat, with the tolerant crop containing higher concentrations of the thiol than the sensitive weeds.[6] It

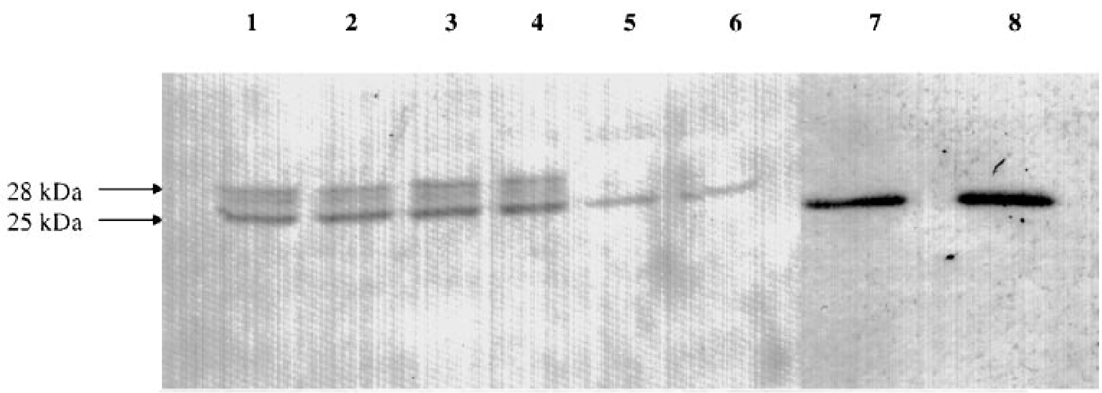


**Fig. 1.** A composite Western blot showing immunoreactive polypeptides resolved by SDS-PAGE and recognised by an anti-GST-serum in extracts from black-grass and wheat. Lanes 1 to 6 show one blot of extracts from 30-day-old black-grass, with lanes 1 and 2 derived from duplicate sets of plants of Lincs E1, lanes 3 and 4 extracts of Peldon and lanes 5 and 6 extracts from Rothamsted. Lanes 7 and 8 are derived from a separate blot of extracts of duplicate samples of 20-day-old wheat.

**TABLE 2**
GST Activities toward CDNB and Relative Levels of a 25-kDa Polypeptide Recognised by an anti-GST Antiserum in the Leaves of Developing Wheat and Black-Grass Plants

| | *GST activity (nkat $g^{-1}$ protein)*[a] *(%intensity)*[b] | | |
|---|---|---|---|
| *Species/Population* | *10 day* | *20 day* | *30 day* |
| Wheat | 3274 (57%) | 5704 (100%) | 5684 (84%) |
| Black-grass | | | |
| Rothamsted | 4673 (8%) | 5122 (10%) | 4776 (8%) |
| Peldon | 13564 (12%) | 11714 (10%) | 13471 (12%) |
| Lincs E1 | 9083 (14%) | 9666 (16%) | 12257 (14%) |

[a] GST activities shown are the means of triplicate determinations, with the standard deviation being less than 3% in each case.
[b] % intensity, determined by probing a single blot with anti GST-antiserum, relative to the 100% intensity shown in the 20-day-old wheat extract.

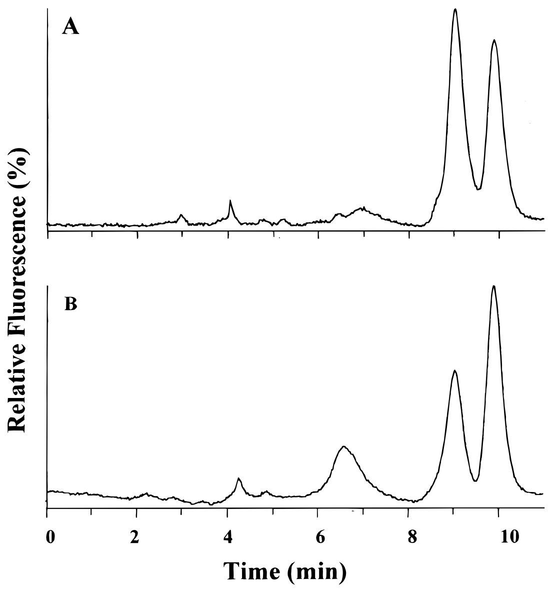


**Fig. 2.** HPLC analysis of fluorescent *S*-bimane derivatives of hydroxymethyl glutathione (retention time 9 min) and glutathione (9·9 min) in extracts from A. 20-day-old wheat and B. 30-day-old black-grass (Rothamsted).

was therefore of interest to determine the available thiols in the differing black-grass populations and compare the contents with those of wheat. Low-molecular-weight thiols were reduced and then *S*-derivatised with bromobimane prior to quantification by HPLC coupled with fluorimetry. HPLC conditions were chosen to resolve the bimane derivative of glutathione from the derivative of hydroxymethylglutathione (gamma-glutamyl-cysteinyl-serine), which has been reported as a major thiol in wheat.[17] HPLC analysis revealed two major fluorescent derivatives in wheat (Fig. 2A). The peak eluting at 9·9 min co-chromatographed with *S*-bimane-glutathione, while, from its relative mobility,[17] the peak eluting at 9·0 min was tentatively identified as *S*-bimane-hydroxymethylglutathione. Black-grass was also found to contain the same two thiols (Fig. 2B) and their concentrations were determined in 30-day-old plants of the three populations and compared with the levels in 20-day-old wheat (Table 3). In wheat, the putative hydroxymethylglutathione was the major thiol, while

**TABLE 3**
Thiol Contents in the Foliage of 20-Day-Old Wheat and 30-Day-Old Black-Grass

| | *Thiol content (nmol $g^{-1}$ FW) ($\pm$SD)*[a] | |
|---|---|---|
| *Species/Population* | *Glutathione* | *Hydroxymethylglutathione* |
| Wheat | 121 ($\pm$20) | 151 ($\pm$33) |
| Black-grass | | |
| Rothamsted | 106 ($\pm$31) | 58 ($\pm$17) |
| Peldon | 180 ($\pm$14) | 29 ($\pm$4) |
| Lincs E1 | 126 ($\pm$6) | 28 ($\pm$6) |

[a] Values represent the means of triplicate determinations.

glutathione predominated in black-grass. This difference in relative abundance of the two thiols meant that, while the total thiol (glutathione + hydroxymethylglutathione) content in wheat was greater than in any of the black-grass populations, the availability of glutathione was similar in the weeds and crop. There was no significant difference in the glutathione contents of susceptible Rothamsted and fenoxaprop-ethyl-resistant Lincs, while the Peldon population contained significantly more glutathione than the other black-grass populations.

## 4 DISCUSSION

There is little information available on GSTs and their role in selectivity in wheat. This presumably results from the relatively few herbicides, such as fenoxaprop-ethyl,[5] dimethenamid[9] and chloroacetanilides,[10] so far shown to undergo GSH-mediated detoxification in wheat. In this crop, the selective use of fenoxaprop-ethyl and dimethenamid is associated with the use of the herbicide safeners fenchlorazole-ethyl[5] and fluxofenim,[9] respectively. In both cases these safeners accelerate the GSH-mediated detoxification of the herbicide in the crop and help prevent phytotoxicity.[5,9] In the case of selectively controlling black-grass, our studies may partly explain the necessity for safeners when using these herbicides. Thus, black-grass shows higher GST activities toward chloroacetanilide and diphenyl ether herbicides than does untreated wheat (Table 1) and has similar levels of glutathione available for detoxification (Table 3). Selectivity based on glutathione conjugation would therefore require the selective increase in GST activities and glutathione content associated with safener treatment in wheat.[7] Herbicide safeners are normally only associated with the induction of GSTs in cereal crops,[4] but there is a report that flurazole, fenchlorim and benoxacor all increase GST activity toward CDNB in black-grass.[18] It will now be of interest to determine the effect of safeners on GST activities toward herbicides in the differing black-grass populations. In contrast to wheat, in the absence of safeners, maize seedlings contain ten-fold higher GST activities toward herbicides than competing weeds of equivalent age and there is a lesser requirement for safeners when using herbicides which undergo glutathione conjugation.[3]

Unlike other weeds of wheat, which reportedly contain much lower levels of glutathione than the crop,[6] black-grass was found to contain similar concentrations of the thiol (Fig. 2 and Table 3). However, wheat differed from black-grass in containing large amounts of an alternative thiol tentatively identified as hydroxymethylglutathione. The importance of hydroxymethylglutathione in herbicide conjugation in wheat and related species warrants further investigation. Conjugates of glutathione, rather than hydroxymethylglutathione, have been identified in wheat plants fed with radiolabelled fenoxaprop[5] and dimethenamid.[9] It will now be of interest to determine whether or not the GSTs of wheat and other panicoid grasses can utilise hydroxymethylglutathione as well as glutathione to detoxify herbicides.

Our results demonstrate that herbicide-resistant populations of black-grass contain elevated levels of GST activity toward fenoxaprop-ethyl, fluorodifen and metolachlor compared with the susceptible Rothamsted strain (Table 1). It has been reported recently that GST activity toward CDNB was higher in Peldon than in Rothamsted, but the relative activities toward herbicides were not determined.[18] Using the wheat anti-GST-serum, Western blot analysis suggested that the enhancement in GST activities in the resistant black-grass populations was due to both increased levels of expression of a constitutive GST, as well as the expression of two novel immunoreactive polypeptides (Fig. 1). The increased expression of GSTs which are normally observed in susceptible weeds in herbicide-resistant weed biotypes has been previously reported in atrazine-resistant *Abutilon theophrasti*.[12] However, the presence of GST subunits in herbicide-resistant weed populations which are below the limit of detection in susceptible individuals grown under identical conditions has not been reported previously. Although expressed in the herbicide-resistant black-grass, it is unlikely that these GSTs are truly unique to these populations and are more likely to represent GST subunits which, though not expressed constitutively in healthy wild-type plants, accumulate during specific phases of development, or in response to stress or environmental cues.[1,4] Various explanations can be proposed to account for the expression of these 'novel' GSTs in resistant populations of black-grass, including mutations which affect relative rates of the transcription and processing of GST coding sequences, or changes in the rates of synthesis and turnover of the respective GST isoenzymes. A definitive explanation awaits the characterisation of the corresponding isoenzymes and genes in herbicide-resistant and -susceptible black-grass.

No definitive role can be ascribed to the importance of modified GST expression on the resistance of black-grass toward herbicides such as fenoxaprop-ethyl, which are detoxified by glutathione conjugation. The Lincs E1 population, which is highly resistant to fenoxaprop-ethyl, contained GST activity toward this herbicide, while the activity was absent in the susceptible Rothamsted strain. However, the Peldon population, which is only partially resistant to fenoxaprop-ethyl, contained similar GST activities to this herbicide as the much more resistant Lincs E1 population. Work on a larger number of herbicide-resistant populations of black-grass is required before any statistically meaningful correlations between GST activity and resistance to

herbicides like fenoxaprop-ethyl can be made. However, our results lend further weight to the suggestion that the development of herbicide resistance in weeds can evolve through the enhancement of a battery of detoxifying enzymes, including mixed-function oxidases and GSTs.[19]

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